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MAPLE SIRUP. X. EFFECT OF CONTROLLED FERMENTATION OF MAPLE SAP ON THE COLOR AND FLAVOR OF MAPLE SIRUP [a]

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Maple sirups produced during the early periods of the maple season are characteristically light amber in color and delicately flavored; it is this type of sirup that is highly valued. As the season advances the sirup becomes progressively darker and acquires a stronger flavor which may not be entirely due to maple. These changes have been mainly associated with the growth of microorganisms in the sap from which the sirup is made *(2, 3, 4)*. The association of color of maple products with quality has been so emphatic that color has become the chief criterion for grading maple sirup.

Studies at this laboratory on the flavor of maple sirup have shown that the desirable flavor component can be increased several fold by heat treatment under special conditions. This indicated that maple sap contains flavor and color precursors which are modified during the processing of the sap to sirup. Although this modification is generally induced by heat during the processing procedure, it could also be influenced by microbiological activity in the sap. These studies were made to determine whether or not desirable changes in flavor and color of maple sirup can result from the fermentation of sap by individual species or combinations of various species of microorganisms. This work establishes that maple flavor can be intensified through proper fermentation of the sap.

MATERIALS, METHODS, AND PROCEDURE

Materials. Maple sap used in these studies was collected under aseptic conditions using the technique described in a previous publication *(4)*. Sap from each sterile 5-gallon collecting bottle was aseptically dispensed into sterile 1-gallon metal cans, quick-frozen, and stored at −29° C. Sterility of the collected sap was determined by sampling each collecting bottle and plating the samples in nutrient agar (7). Any lot of sap having a count exceeding one colony per ml. was discarded. Since all of the fermentations could not be conducted simultaneously, they were made at 4 different times with four lots of sap. Careful selection of sap was required so that identical replicates could be used throughout the 4 parts of the study. To obtain this uniformity, sixteen 4-gallon collections of sterile sap were selected. One gallon of sap from each collection was allocated to each of the 4 lots so that at the completion of the allocation each 16-gallon lot contained equal volumes of sap from the same trees and the same flows.

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To prepare each lot of frozen sap for use in fermentation studies, a procedure was developed to thaw the sap rapidly in a manner which minimized microbial contamination and chemical changes. The desired amount of frozen sap was removed from the gallon cans, crushed in a sanitized power crusher and the crushed ice melted rapidly in a steam jacketed kettle. To prevent changes in the sap during heating, the kettle was charged with about one gallon of the crushed frozen sap, heated with steam at 5 p.s.i. and the ice slurried rapidly until the greatest portion had melted, at which time the sap was removed. The residual ice melted in a short time and the final temperature of the sap was 15-20° C. The melted sap was blended and dispensed into sterile 50-liter carboys for fermentation.

Cultures. Four strains of bacteria and one strain of yeast, all isolated from naturally fermenting sap, were used in these studies. All of the bacterial strains were aerobic, gram negative rods capable of developing good growth at temperatures of 2-3° C. Two of the cultures develop light cream colored colonies and produce a bluish-green water soluble pigment. The cells of these two cultures are very motile, having 3 to 5 flagella at one pole. They produce proteolysis of milk and liquefaction of gelatin. Preliminary taxonomic studies *(7)* place these two organisms in the genus *Pseudomonas*. One of these cultures is designated as *Pseudomonas*-11 and the other *Pseudomonas*-25. A third culture produces a yellow pigmented colony and the cells are non-flagellate. It produces proteolysis when cultured in milk and causes liquefaction of gelatin. This bacterium is tentatively classified as a species of *Flavobacterium* and is designated here as *Flavobacterium*-583. The fourth culture produces colonies with a pink-orange pigmentation. The cells are motile and possess a single polar flagellum The culture does not cause proteolysis of milk or liquefaction of gelatin and at the present time has not been classified but is designated as *Bacterium*-587.

Methods. Refractive index measurements for the determination of per cent solids as sucrose (degrees Brix) were made at 20° C. with the Abbe refractometer. Color indices [c] *(6)* were determined from absorption measurements at 450 mμ made on sirups of 65-67° Brix pressure filtered through three layers of Whatman [d] No. 52 filter paper in a Seitz filter. Per cent of invert sugar was determined by the Berlin method *(5)*. Bacterial counts were made by plating appropriate dilutions of sap samples on nutrient agar and yeast counts were made on Wickerham's Agar *(9)*. Plates were incubated at room temperature for 48 hours. Flavor evaluations of the sirups were made by an experienced panel.

Procedure. The study consisted of 8 separate fermentations, 5 being produced by specific strains of microorganisms and 3 by combinations of specific bacteria and a yeast. The collected sap for these fermentations was taken from frozen storage in 4 successive lots of 16 gallons each (as described above) and thawed. Two gallons of each lot were retained for conversion to the control sirup, and the remaining 14 gallons were divided and dispensed into two sterile carboys for inoculation. The procedure was repeated for the remaining 6 fermentations.

Inocula were prepared from actively growing cultures by washing the organisms from agar slants and making appropriate dilutions. Where combinations of organisms were used for inoculations, the washings from the selected slants were combined to prepare the inoculum. The number of organisms in the inoculum for each fermentation was determined from plate counts of sap samples taken immediately after inoculation.

The inoculated sap was incubated in a cold room at 0.5-2.5° C. which approximates temperature conditions occurring in the field during the sap season. At the end of 3, 6, and 10 days of incubation, samples were withdrawn from each inoculated carboy for plate counts and 2-gallon portions were removed aseptically for conversion to sirup.

The 2-gallon portions of sap were reduced to sirup using the following standardized conditions selected to simulate the conditions occurring in a commercial evaporator. Using a steam-jacketed kettle, the sap was evaporated rapidly (30-35 minutes) to a

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[c] Color index, $A^{86.3\%}_{1\,cm.} = A^{1}_{450}$ (86.3/bc), where A_{450} is the observed absorbance at 450 mμ, b is the depth of solution in centimeters, and c is the grams of solids as sucrose per 100 ml.

[d] Mention of commercial products does not imply endorsement by the Department of Agriculture over others of similar nature not mentioned.

volume calculated to give a density of approximately 45° Brix. This sirup was transferred to a stainless steel beaker equipped with a condenser and refluxed for one hour over a Meeker burner. The refluxed sirup was transferred to a 1-quart steam kettle and evaporated rapidly (5-8 minutes) to standard density sirup (65.5° Brix).

The following determinations were made on the sirups prepared from the fermented sap: per cent of invert sugar, color indices, and flavor evaluations.

RESULTS AND DISCUSSION

Table 1 shows the growth of microorganisms in maple sap. Increase in number of microorganisms is found in all cases except one where the combination of *Bacterium*-587 and yeast shows a decrease after the third day of incubation. The increased growth at these low temperatures establishes

TABLE 1

Growth of microorganisms inoculated into maple sap

Organisms	Number of microorganisms per ml. of sap			
	Days of incubation			
	0	3	6	10
Pseudomonas-25	1,090,000	18,300,000	lost	58,000,000
Bacterium-587	1,040,000	3,100,000	2,500,000	8,000,000
Yeast	31,000	390,000	1,580,000	4,600,000
Pseudomonas-11	1,440,000	lost	25,000,000	59,000,000
Flavobacterium-583	310,000	1,610,000	3,100,000	2,770,000
Pseudomonas-11 and Yeast	850,000	lost	5,500,000	52,000,000
Flavobacterium-583 and Yeast	330,000	1,400,000	7,400,000	12,200,000
Bacterium-587 and Yeast	4,300,000	8,500,000	5,100,000	3,700,000

that maple sap is a good growth medium for these adventitious organisms; this is especially noticeable in the case of the species of *Pseudomonas* where growth increased approximately 60 fold.

The changes in maple sirup produced from sap that had been fermented with 8 different inocula are presented in Table 2. These include development of flavor, color, and free reducing sugars. Flavors were of two types: a full-bodied maple flavor and an acrid caramel flavor. Levels of maple flavor are expressed on a scale of 4, with 1 corresponding to the flavor level present in a commercial U. S. grade AA maple sirup, and 2, 3, and 4 indicating increments of increasing intensities. In the case of caramel, 1 designates the lowest detectable level and levels of increasing intensities are designated by 2, 3, and 4 where 4 represents a strong caramel flavor.

With one exception, all of the sirups made from sap fermented by these organisms had a distinctive maple flavor with an intensity level of 1 or more. Fermentation of sap by *Pseudomonas*-25 produced the greatest effect. The flavor level attained a score of 2 after only 3 days of fermentation, and was increased to a score of 3 and 4 after 6 and 10 days, respectively. The unusual nature of this fermentation was the development of a high level of maple flavor without the production of caramel, although the color of the sirup was greatly increased. In commercial practice, the production of a dark colored sirup (U. S. unclassified) rich in maple flavor and free of caramel is never encountered. Another unusual characteristic of the sirup resulting from this fermentation was the lack of any residual

TABLE 2

Properties of maple sirup produced from sap fermented by various microorganisms

	Increase in color index over control			Change in per cent invert sugar over control			Flavor [1]					
							Maple			Caramel		
Days incubated	3	6	10	3	6	10	3	6	10	3	6	10
Organism:												
Pseudomonas-25	0.22	0.61	2.15	0.0	0.0	+0.1	2	3	4	0	0	0
Bacterium-587	0	0.19	0.28	0	lost	0.0	1	1	1	0	0	0
Yeast	0.03	0.43	1.78	0.0	+0.3	+5.2	1	1	1	0	1	4
Pseudomonas-11	0.35	0.47	2.11	+0.1	+0.1	+0.1	1	1	1	0	0	1
Flavobacterium-583	0.20	0.52	1.15	+0.0	+0.4	+0.6	2	2	2	0	1	2
Pseudomonas-11 and Yeast	0.45	0.81	2.78	+0.1	+0.1	+0.3	1	2	2	0	0	2
Flavobacterium-583 and Yeast	0.15	1.09	1.49	+0.1	+1.4	+2.2	2	2	2	0	2	4
Bacterium-587 and Yeast	0.09	0.29	1.07	+0.0	+0.2	+1.0	off-flavor, increasing with time					

[1] Flavor has been given numerical values of 0, 1, 2, 3, and 4 with 0 being lack of the particular flavor and 4 being the greatest amount. In the case of maple, 1 is indicative of good maple flavor as exhibited in the control sirup.

reducing sugars. In commerce all strongly flavored, dark-colored sirups (U. S. grade B or below) contain relatively large amounts of free reducing sugars.

Sap fermented with *Bacterium*-587, like that fermented by the *Pseudomonas*-25, yielded sirup that contained no caramel flavor and did not cause the formation of any free reducing sugars. This sap did not, however, yield a sirup of increased maple flavor even after the 10 days of fermentation. It should be noted that this organism did not develop a high population in this medium and its total population after 10 days incubation was only one-tenth that of *Pseudomonas*-25.

The effect on flavor of fermenting sap with *Pseudomonas*-11 was much the same as that caused by *Bacterium*-587 except that a trace of caramel flavor was detected after 10 days' incubation. The growth rate of this species of *Pseudomonas* was very similar to that of the other representative of this genus.

The sirup made from sap fermented by yeast showed the development of a dark color and a strong caramel flavor accompanied by a large increase in free reducing sugars. This is in agreement with current observations *(8)* which indicate that in heated sugar solutions the amount of color developed is related to the amount of reducing sugars present.

The flavor formed in sirup produced from sap fermented by *Flavobacterium*-583 was intermediate between that caused by *Pseudomonas*-25 and by yeast. The level of maple and caramel flavors was only one-half that caused by the *Pseudomonas* and yeast, respectively. The rate of maple flavor development in the case of *Flavobacterium* was more rapid since the sirup made from sap fermented 3 days had a maple flavor level of 2 as compared to a level of 1 for other fermentations with the exception of *Pseudomonas*-25. The *Flavobacterium* caused the formation of some reducing sugars. This could account for the production of caramel flavor in the sirup but not for the lighter color which was only one-half that formed

in the *Pseudomonas* fermentation. Failure of the *Flavobacterium* to cause the production of large amounts of maple and caramel flavors may be due to its slower growth rate in sap. Its population at the end of 10 days of incubation was only one-twentieth of that produced by the more rapidly growing *Pseudomonas* species.

In the natural uncontrolled fermentation of maple sap, it is doubtful that fermentation is ever caused by a pure culture. Therefore, fermentations using mixed cultures were conducted. For simplicity the mixtures included only two cultures. The yeast culture was used in every mixture because yeasts often appear as one of the predominating groups of organisms late in the season, and it was desired to learn the effect of this culture on flavor and color of sirup when grown in combination with bacteria.

In the yeast-*Pseudomonas*-11 mixture there was a slight increase in the amount of maple flavor produced after 6 days of incubation as compared to that produced by each organism separately, and the amount of caramel produced was diminished over that produced by yeast alone. This may indicate that the rapidly growing *Pseudomonas* retarded the growth of the yeast.

The effect of the mixed culture of *Flavobacterium*-583 and yeast was as would be expected. The slow growing *Flavobacterium* did not retard the growth of the yeast so that the net effect on the sirup was as if each were causing the fermentation independently, the *Flavobacterium* causing a maple flavor level of 2 and the yeast a caramel flavor level of 4.

The effect on flavor of the mixed culture of *Bacterium*-587 and yeast was unexpected. Even after only 3 days of fermentation it imparted to the sirup a pronounced off-flavor which increased with time. This off-flavor was not present in sirup made from sap fermented with either of these organisms separately.

The observation that fermentation of sap by certain microorganisms can induce changes which result in the intensification of the desired flavor may be a clue to the long sought explanation for the question: what makes maple flavor? In addition to these microbial fermentations the effect of culture extractives and enzymes is being investigated. An understanding of the changes which occur will be of great advantage in developing conditions for optimum processing of this product and these studies should ultimately lead to methods which will aid the control of flavor and color in maple sirup.

SUMMARY

This study shows that the fermentation of maple sap has a marked effect on the flavor and color of the sirup produced. Evidence is presented for generic differences in the degree and type of changes produced in the flavor and color. Some species of microorganisms produce intensification of maple flavor only, while others favor the production of caramel flavor or maple with varying degrees of caramel. The caramel flavor of maple sirup is correlated with the amount of free reducing sugars formed during microbial growth in sap, and is especially apparent in the case of yeast fermentation.

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